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The Determination of Optimum Staining Time for Hibiscus Extract Nuclear Staining Using Buffered Formalin Fixed, Paraffin Embedded Lung TissueBenard S.A.^{1*}, Afolabi O.O.², Bankole J.K.³, Okoye J.O.⁴, Fowotade A.A.¹, Olutunde O.A.¹

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Abstract

This exploratory study investigated the optimum nuclear staining time of *H. sabdariffa* aqueous extract mordanted with ferric chloride solution on 10% neutral buffered formalin fixed, paraffin embedded lung tissue. Blocks of lung tissues were retrieved from the post-mortem block archive of the Pathology Department, University of Ilorin Teaching Hospital, Ilorin, Kwara State, Nigeria. The blocks were sectioned at 3-5 microns with a Leica Rotary microtome and stained with Hibiscus-Eosin staining technique for 5 minutes (A), 10 minutes (B), 15 minutes (C), 20 minutes (D), 25 minutes (E), 30 minutes (F) and 60 minutes (G). Parallel staining was done with H&E as control. Stained slides were blindly assessed independently by three Histopathology experts for cell membrane appearance, nuclear membranes, cytoplasm transparency and extracellular matrix. Photomicrographs revealed satisfactory staining by the Hibiscus-eosin technique comparable to standard H&E. Assessment by the Histopathology experts revealed that optimal staining was achieved between 5-10 minutes. The optimum staining time for Hibiscus nuclear staining is 5-10 minutes.

Keywords: Hibiscus, roselle, staining, time, nucleus**Introduction**

Hibiscus is an ideal crop for developing countries as it is relatively easy to grow, can be grown as part of multi-cropping systems and can be used as food and fibre (Ines Da-Costa, 2014). It is an erect, mostly branched, annual shrub. The flowers are large, short-pedunculated, red to yellow with dark center. The most recognized cultivars are 'Victor', 'Rico', and 'Archer'. Botanical variants are var. *sabdariffa*, var. *altissima* and the green form known as the white sorrel. The plant is native to Angola, Egypt, Guatemala, Guinea, India, Myanmar, Nigeria,

Philippines. Exotic countries where the plant is widely distributed include Jamaica, Mexico, Spain and US (Orwa *et al.*, 2009). Documented reports have shown that *H. sabdariffa* is used traditionally in folk medicine as hypotensive agent, antidiuretic, hot or cold beverage and antioxidant (Ines Da Costa *et al.*, 2014). The watery extract has been used as a biological staining agent for some fungal species (Ihuma *et al.*, 2012), used as a dye to demonstrate ova of parasites (Okolie, 2008), used as an antimicrobial agent (Zinatul *et al.*, 2012), applied as a staining agent to demonstrate sperm morphology (Bassey *et al.*, 2012) and histologically as a cytoplasmic and nuclear stain (Muhammed *et al.*, 2016).

The aqueous extract was used as a cytoplasmic stain on kidney tissue as a substitute to eosin (Eman, 2006). Abd-Alhafeez and Colleagues (2014) used it as a histological cytoplasmic stain for intestine and renal tissues. Raheem and Colleagues (2015) too used *H. sabdariffa* extract as a natural histological cytoplasmic stain on skin tissue.

In contrast, Benard (2008) used a mordanted aqueous solution of *H. sabdariffa* as a nuclear stain for lymph node and appendix. Later on, Egbujo and Colleagues (2008) used mordanted solution of *H. sabdariffa* as nuclear stain for testis. Other works toed this line to use *H. sabdariffa* mordanted solution as nuclear stain for brain (Benard *et al.*, 2015), hippocampus (Muhammed *et al.*, 2016), liver and kidney (Benard *et al.*, 2015), skin (Agbede *et al.*, 2017; Benard *et al.*, 2017); muscle and skin (Omorodion and Achukwu, 2017).

Staining time on methods used from available reports varies depending on the method of extraction and target cellular component. The iron-roselle nuclear staining method recommended staining for 5 minutes, however, there is a need to compare and

evaluate staining quality at different staining time as there is paucity of information in the literature in this regard. This work assesses the quality of nuclear staining at different durations with a view to determining the optimum staining time.

Materials and Methods

Preparation of Hibiscus Calyces

Dry calyces of *H. sabdariffa* were purchased at a local market in Ilorin, capital city of Kwara, State, Nigeria and grounded using a Binatone blender to a fairly powdery form. 10g of the red calyces was weighed using an Ohaus digital balance and dissolved in 200ml of distilled water on a Bunsen burner to boiling point to give a brilliant red coloured extract which was immediately allowed to cool and filtered to give a clear filtrate. The hibiscus-iron staining technique of Benard and Colleagues (2016) was adopted and used to stain the sections with H&E as parallel control.

Formulation of Extract Solution

To 100ml of the clear extract, 5g sodium chloride, 1.2ml of 10% Ferric chloride solution and 3ml of glacial acetic acid were added, mixed properly in a brown bottle and kept in a refrigerator at 4°C.

Preparation of Tissue Blocks

Blocks of lung tissues were retrieved from the post-mortem block archive of the Pathology Department, University of Ilorin Teaching Hospital, Ilorin, Kwara State, Nigeria. The blocks were sectioned at 3-5 microns with a Leica Rotary microtome. Sections were grouped into 7 categories and labeled A-G to

reflect staining time variations of 5 minutes (A), 10 minutes (B), 15 minutes (C), 20 minutes (D), 25 minutes (E), 30 minutes (F) and 60 minutes (G) respectively.

H&E Staining Method

Sections were dewaxed in Xylene and hydrated through 100%, 90%, 70% and 50% alcohol to water. They were subsequently stained in Harris haematoxylin for 15 minutes, washed in running water for 2 minutes, differentiated in 1% acid alcohol, blued in running tap water for 10 minutes, counterstained in 1% alcoholic eosin for 1 minute, dehydrated in ascending grades of alcohol, cleared in xylene and finally mounted in DPX.

Hibiscus–Iron Staining Method

Sections were dewaxed in xylene and hydrated through 100%, 90%, 70% and 50% alcohol to water. They were subsequently stained in Hibiscus extract solution for 5 minutes, washed in running water for 2 minutes, counterstained in 1% alcoholic eosin for 1 minute, dehydrated in ascending grades of alcohol, cleared in xylene and finally mounted in DPX.

Result

Nucleus stains purple-blue, cytoplasm, pink and red blood cells, red in similar manner as standard haematoxylin and eosin. Assessment by independent experts shows good staining at 5 and 10 minutes. Staining at 15, 20, 25, 30 and 60 minutes were adjudged to be satisfactory.

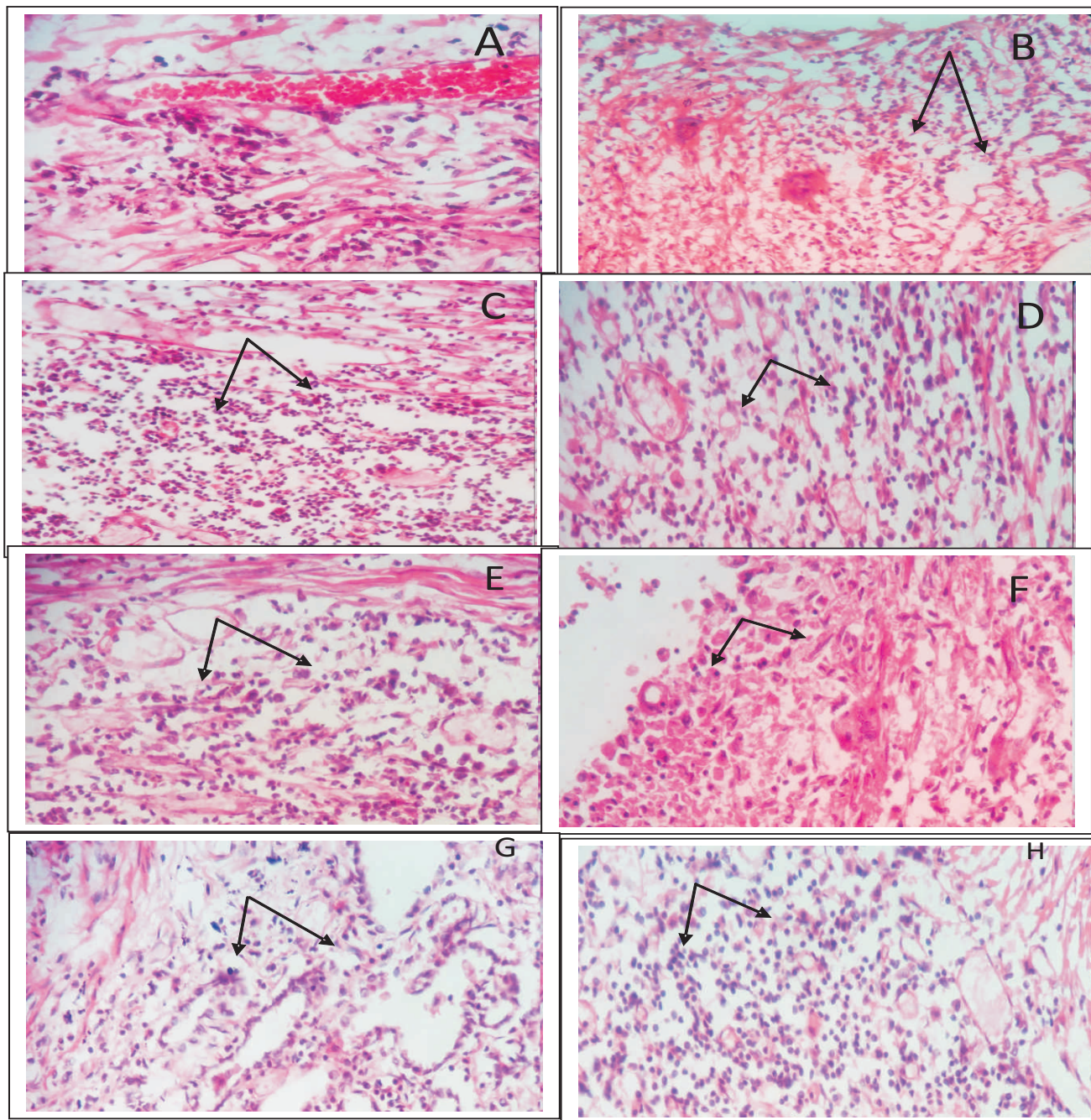


Figure A- H&E Control, B: Hib-Eosin 5mins; C: Hibiscus-Eosin 10 mins; D: Hibiscus-Eosin 15 min; E: Hibiscus-Eosin 20mins; F: Hibiscus-Eosin 25 mins; G: Hibiscus-Eosin 30 mins; and H: Hibiscus-Eosin 60 mins staining of lung tissue. Mag. X 200

Discussion

Previous efforts on *H. sabdariffa* nuclear staining has been on the suitability of the extract to substitute for haematoxylin in histological staining techniques. This current effort is to methodologically derive an optimum staining time for *H. sabdariffa* iron mordanted solution as a nuclear stain to determine the propriety of it being used as a progressive or regressive stain.

The sections were assessed on nuclear appearance, cell membrane, cytoplasm transparency and extracellular stroma. Sections stained for 5

minutes were graded good (++) for demonstrating the nuclear and cytoplasm components beyond satisfaction (Fig. B). Sections stained for 10 minutes (Fig. C) were also graded good (++)). However, sections stained for 15 minutes, 20 minutes, 25 minutes, 30 minutes and 60 minutes (Fig. D, E, F, G and H) were graded as satisfactory (+). It could be deduced from this results that optimal staining could be attained between 5-10 minutes. This supports previous use of the staining technique as a progressive dye (Agbede et al., 2017; Benard et al., 2016; Muhammed et al., 2016). This time advantage

recommends the staining technique as a veritable alternative to haematoxylin especially in season of scarcity bearing in mind that the staining time of routine Harris haematoxylin is 15-20 minutes. Observers looking at the microscope rarely question the Hibiscus-Eosin stained sections as they often mistake it for H&E. Future efforts to investigate the optimum staining time in a panel of organs simultaneously is underway to further expose comparative results for optimal staining time assessment. Optimal staining time of 5-10 minutes is established for Hibiscus-Eosin nuclear staining especially for lung tissues.

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